The interaction of the cell-contact proteins VASP and vinculin is regulated by phosphatidylinositol-4,5-bisphosphate

Stefan Hüttelmaier*, Oleg Mayboroda†, Birgit Harbeck*, Thomas Jarchau‡, Brigitte M. Jockusch* and Manfred Rüdiger*

Background: Focal adhesion sites are cell–matrix contacts that are regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2)-dependent pathways. Vinculin is a major structural component of these sites and is thought to be engaged in multiple ligand interactions at the cytoplasmic face of these contacts. Cytoplasmic vinculin is considered to be inactive due to its closed conformation involving intramolecular head–tail interactions. Recently, the vasodilator-stimulated phosphoprotein (VASP), a substrate of cyclic AMP-dependent or cyclic GMP-dependent kinases and a component of focal adhesion sites, was shown to bind to vinculin.

Results: VASP–vinculin complexes could be immunoprecipitated from cell lysates and, using immunofluorescence, both proteins were found to colocalize in nascent focal adhesions. Consistent with the view that vinculin must be activated at these sites, we found that PIP_2, levels of which are elevated during the early stages of adhesion, bound to two discrete regions in the vinculin tail, disrupting the intramolecular head–tail interaction and inducing vinculin oligomerization. Vinculin–VASP complex formation was greatly enhanced by PIP_2 and both the EVH1 and EVH2 domains of VASP participated in vinculin binding.

Conclusions: Focal contact assembly involves interaction between VASP and vinculin, which is enhanced by PIP_2-induced vinculin activation and oligomerization. Given that vinculin and VASP both bind to F-actin, vinculin–VASP complexes might bundle the distal ends of actin filaments in focal contacts. We propose that PIP_2-dependent signalling modulates microfilament organization at cellular adhesion sites by regulating vinculin–VASP complexes.

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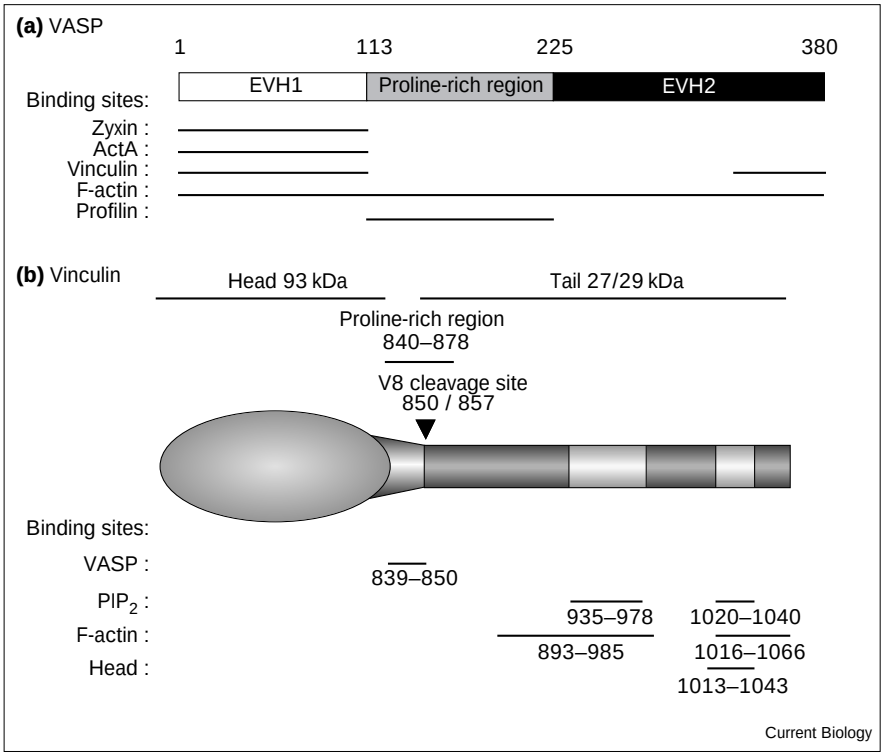
Background

Vasodilator-stimulated phosphoprotein (VASP), a protein of 380 amino acids, is a substrate of cyclic AMP-dependent and cyclic GMP-dependent protein kinases. It is found in a variety of tissues and is associated with focal adhesions, microfilaments, cell–cell contacts and the lamellipodia of cultured cells [1–4]. Recent data suggest that VASP has an important role in the regulated organization of microfilaments at membrane-attachment sites in general, including the interface between the bacterium *Listeria monocytogenes* and the actin comet tail recruited by this intracellular parasite from the host's actin supply [5,6]. VASP has been shown to be a multi-ligand protein that binds directly to filamentous actin [2], profilin [7], the *Listeria* surface protein ActA [6], zyxin [3] and vinculin [8,9]. Like zyxin [10,11], vinculin, a 116 kDa protein, is a major component of cellular junctions [12,13]. Assembly, maintenance and regulation of adherens junctions and thus tissue integrity depend critically on vinculin levels [14–16]. The overall domain structures of vinculin and its ligand VASP are depicted in Figure 1. The binding of VASP to vinculin, ActA and zyxin has been attributed to proline-rich sequences, in particular to an FPPPP (FP_4; in single-letter amino-acid code) motif present in all three of these ligands [8,9,17,18]. Whereas binding of VASP and related proteins [19] to zyxin and ActA is of rather high affinity, binding to vinculin was found to be comparatively weak. This was explained by the fact that zyxin and ActA contain tandem repeats of the FP_4 motif, whereas vinculin contains only a single copy [9]. In addition, VASP–vinculin complex formation may be influenced by an intramolecular interaction of the vinculin head and tail domains that controls the activity of ligand-binding sites [20], as already demonstrated for vinculin binding to F-actin, talin, α-actinin and acidic phospholipids [21–26]. Recently, it has been postulated that this intramolecular interaction and thus ligand binding of vinculin might be regulated by binding of the signalling molecule phosphatidylinositol-4,5-bisphosphate (PIP_2) and possibly also by serine/threonine phosphorylation [27–30].

Here, we show that both VASP and vinculin concentrate in focal contacts during cell spreading. For several mammalian

Figure 1



Schematic organization of VASP and vinculin. These illustrations are based on references given in the text. **(a)** VASP can be divided into three functionally distinct domains: EVH1, the proline-rich central domain and EVH2, each delineated by the labelled amino-acid residues. The location of the binding sites for identified ligands are indicated. The F-actin-binding region has not yet been attributed to one of these domains. VASP exists as a tetramer, but structural details have not been elucidated. **(b)** Vinculin comprises a large, compact amino-terminal head and a rod-like tail that are connected by a flexible, proline-rich hinge region. The FP_4 motif is located within the proline-rich region. A V8 cleavage site severs the larger part of the hinge region plus the tail from the head. Binding sites for various ligands of the head, hinge and tail domains are indicated.

cell types, the contact between integrins and the extracellular matrix, which is established during spreading, has been shown to increase synthesis of PIP_2, resulting in elevated PIP_2 levels [31,32]. We demonstrate that PIP_2 activates vinculin by interfering with its head–tail association, favouring its oligomerization. We have characterized sites in the vinculin tail that are involved in PIP_2 binding and show that PIP_2 binding greatly enhances formation of the VASP–vinculin complex, probably stimulated by vinculin oligomerization. On the basis of these data, we propose a model of how these molecules might be involved in the organization of the actin cytoskeleton during the assembly of cell–matrix attachment sites.

Results

Vinculin and VASP form complexes in cells and colocalize in nascent focal contacts

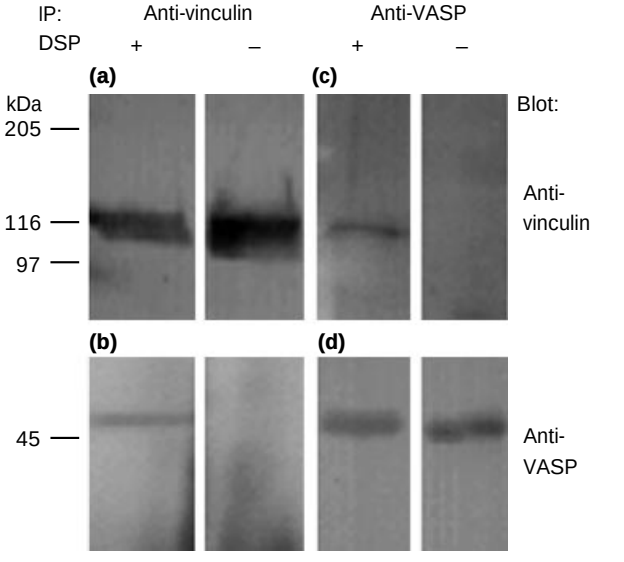
VASP, like vinculin, is a prominent component of cellular adherens junctions [2,7,13]. Both VASP and vinculin colocalize in fully spread, stationary epithelial and fibroblastic cells, suggesting that the direct interaction of both proteins shown *in vitro* [8,9] is also relevant in living cells. To detect vinculin–VASP complexes under physiological conditions, we used chemical cross-linking of proteins in cells, before solubilizing protein aggregates in a lysis buffer. HeLa cells were treated with the membrane-permeable cross-linker dithiobis-succinimidylpropionate (DSP), and lysed 30 minutes later. The extracts were incubated with either anti-vinculin or anti-VASP antibodies, and both sets of immunoprecipitates were analyzed in immunoblots with antibodies against both proteins. As seen in Figure 2, the immunoprecipitates obtained with either anti-vinculin or anti-VASP antibodies contained both vinculin and VASP. Thus, these results suggest the presence of vinculin–VASP complexes in living cells.

Both VASP and vinculin are recruited at an early stage during the formation of focal contacts in spreading cells. HeLa cells, fixed and processed for detection of VASP and vinculin by immunofluorescence 0.5–1 hours after seeding, showed peripheral lamellae and multiple small focal contacts. As seen in Figure 3, both VASP and vinculin were concentrated in the small peripheral focal contacts formed during cell spreading 1 hour after seeding. Identical results were also obtained with cells 30 minutes after seeding and with cells transfected with a green fluorescent protein (GFP)–VASP fusion construct (data not shown). Hence, these observations indicate that vinculin and VASP are already found in a complex in living cells when PIP_2 levels are elevated [31,32].

PIP_2 disrupts head–tail interactions and induces tail oligomerization in vinculin

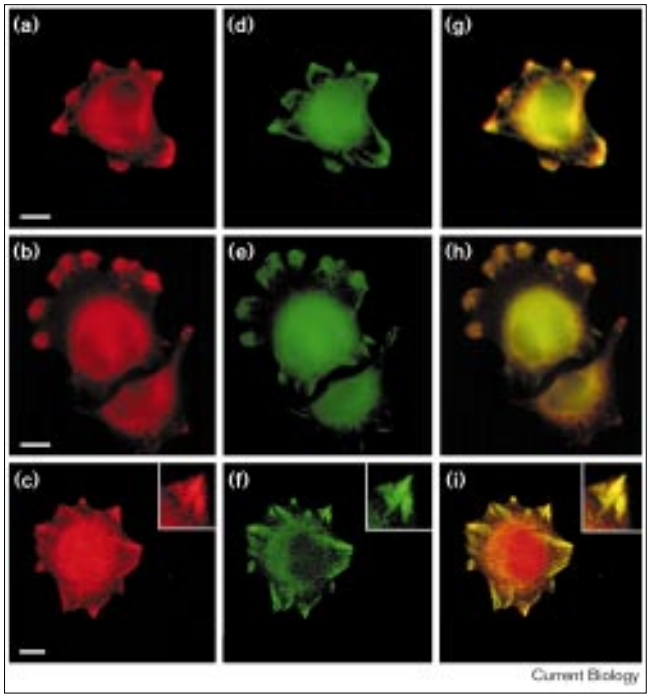
Current evidence suggests that cytoplasmic vinculin is inactive in ligand binding, because it has a closed conformation, but becomes activated at adhesion sites [25].

Figure 2



Complex formation between VASP and vinculin in cells, as detected by immunoprecipitation after *in situ* cross-linking. HeLa cells either treated (+) or not treated (–) with DSP were lysed and subjected to immunoprecipitation (IP) with **(a,b)** anti-vinculin or **(c,d)** anti-VASP antibodies. Both precipitates were analyzed by SDS–PAGE and immunoblotted with either (a,c) anti-vinculin or (b,d) anti-VASP antibodies. Immunoblots were developed with either (a,d) chloronaphthol or (b,c) enhanced chemiluminescence. Note that VASP–vinculin complexes were detected only after *in situ* cross-linking.

Figure 3



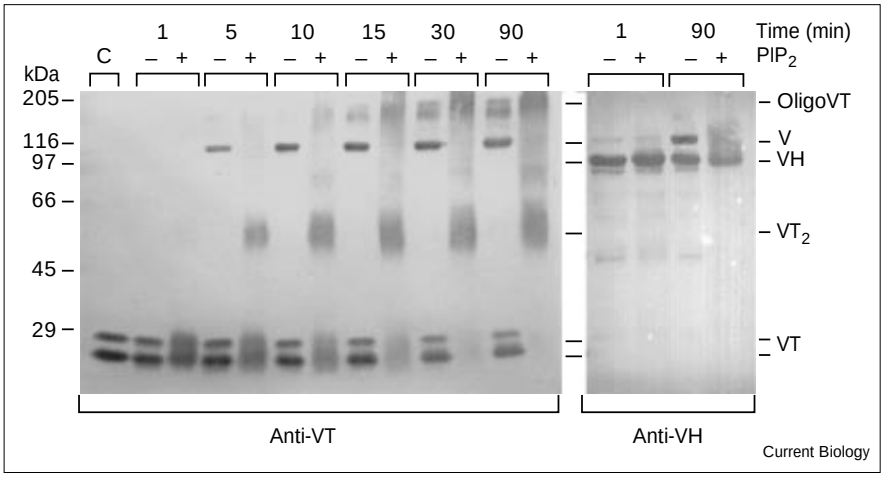
Recruitment of VASP and vinculin to nascent focal contacts in spreading cells. HeLa cells were fixed 1 h after seeding and double-stained with antibodies against VASP (red) and vinculin (green). At this early time point after seeding, the attached cells are still fairly globular, but at the periphery, protruding lamellae have formed with small, nascent focal contacts which contain both **(a–c)** VASP (red) and **(d–f)** vinculin (green), as seen in **(g–i)** the superimposed images (yellow). (a,b,d,e,g,h) Conventional fluorescence microscope images of two different cells. (c,f,i) Images obtained by confocal laser scanning microscopy. The inset shows an enlargement of a nascent focal contact. Bars represent 10 μm, the insets are magnified 2.5 times.

Activation of vinculin may involve the release of its head–tail interaction by PIP_2 [27,29]. To characterize further the effect of PIP_2 on vinculin head–tail interactions and to characterize the PIP_2-binding site in vinculin, we performed cross-linking experiments with isolated head (amino-acid residues 1–850) and tail (amino-acid residues 851–1066 and 858–1066) fragments and monitored the effect with antibodies specific for either the vinculin head or tail regions. In the absence of PIP_2, mixtures of isolated vinculin heads and tails (molar ratios 1:1) were rapidly and efficiently cross-linked by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), as demonstrated by the fact that, after cross-linking, the tail-specific antibody recognized a target of the same molecular weight as intact vinculin (Figure 4). The proportion of cross-linked fragments increased with time over 90 minutes (Figure 4). In contrast, in the presence of 100-fold molar excess PIP_2, the tail fragments showed no such shift. Instead, a new antibody-reactive band appeared at the calculated position of the vinculin-tail dimer and, at later time points, increasing amounts of oligomeric complexes composed only of vinculin tails were seen, whereas the monomeric tail species had disappeared (Figure 4). Hence, PIP_2 efficiently inhibits the head–tail interaction of vinculin and induces oligomerization of the tail fragments.

The specificity of this effect was studied further. Figure 5 shows the concentration-dependent oligomerization of vinculin tail fragments induced by either PIP_2 or phosphatidylserine (PS), again seen after EDC cross-linking and monitored with the tail-specific anti-vinculin antibody. At molar ratios of 1:1 to 1:100 (protein : phospholipid), PIP_2 induced the formation of oligomers increasing in size and quantity, leading to a decrease in the amount of the monomeric form, whereas PS was much less effective, yielding no substantial decrease of the monomer pool (Figure 5). The formation of higher molecular weight vinculin–tail oligomers (trimers to pentamers) was most efficient within the PIP_2 molar-excess range of 1:10 to 1:20, whereas above this value, still more dimers were formed but the formation of larger oligomers was seen to decline. Such an effect was not seen with PS over the same concentration range. Analogous curves showing optimum protein

Figure 4

The effect of PIP_2 on head–tail interaction in vinculin. Head and tail fragments of vinculin were cross-linked with EDC in the absence (–) or presence (+) of a 100-fold molar excess of PIP_2 for between 1 and 90 min as indicated. Vinculin tail fragments (VT), tail dimers (VT_2), full-length vinculin (V) and vinculin tail oligomers (oligoVT) were identified with a vinculin-tail-specific antibody (anti-VT; left panel), and vinculin head (VH) and full-length vinculin (V) were detected with a vinculin-head-specific antibody (anti-VH; right panel). C indicates control sample without the cross-linker. After 30 min all vinculin tail monomers have been cross-linked in the presence of PIP_2.



to phospholipid ratios have been described previously for PIP_2 binding to α-actinin [33]. Similar results were obtained with full-length vinculin and with the PIP_2-binding fusion proteins described in Figure 6 (data not shown).

To determine whether other phospholipid components of plasma membranes might influence the effect of PIP_2 on vinculin, we analyzed PIP_2-induced vinculin oligomerization in the presence of phosphatidylcholine (PC). Cross-linking experiments and subsequent analysis of the samples by SDS–PAGE, analogous to the experiments described in Figure 4, showed that the stimulation of oligomer formation was completely inhibited by the presence of 10% PC in the lipid mixture (data not shown). Thus, in this system, we observed activation of vinculin only upon PIP_2 binding.

PIP_2 binds in the same region as actin in the vinculin tail

We next identified PIP_2-binding regions within the vinculin tail. Various vinculin tail fragments, produced as maltose-binding protein (MBP) fusion proteins in *Escherichia coli*, full-length purified vinculin and the proteolytically cleaved vinculin tail were each tested for PIP_2 binding by immunoprecipitation with anti-PIP_2 antibody. As seen in Figure 6, a small amount of protein precipitated without PIP_2 due to non-specific binding to protein-A–Sepharose. In the presence of PIP_2, however, vinculin and its tail fragment, and various recombinant fusion proteins containing tail sequences, were efficiently precipitated by the PIP_2 antibody. MBP alone did not sediment, in either the presence or the absence of PIP_2. The data suggest that two separate sequence stretches of vinculin (amino acids 893–985 and amino acids 1016–1066) are

Figure 5

The effect of PIP_2 and phosphatidylserine (PS) on vinculin oligomerization. Vinculin tail fragments were cross-linked by EDC in the presence of increasing amounts (0–100-fold molar excess) of either **(a)** PIP_2 or **(b)** PS. Oligomerization, indicated by the appearance of cross-linked dimers and oligomers, was followed with the vinculin-tail-specific antibody, and was more efficient in the presence of PIP_2 compared with PS, showing an optimum at a 10–20-fold excess. PL indicates phospholipid; EDC, cross-linker. The numbers refer to the molar excess of the phospholipid with respect to protein. The calculated positions for vinculin tail monomers (VT), tail dimers, trimers, tetramers and pentamers are indicated on the right.

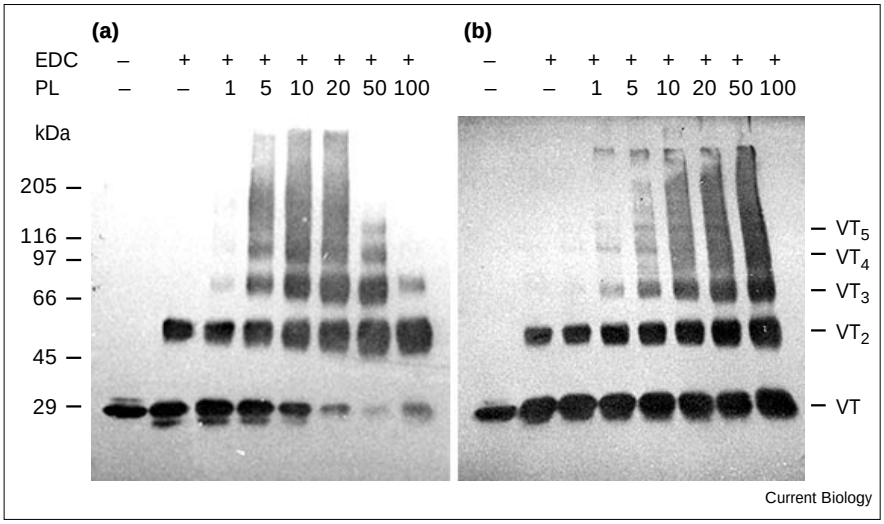
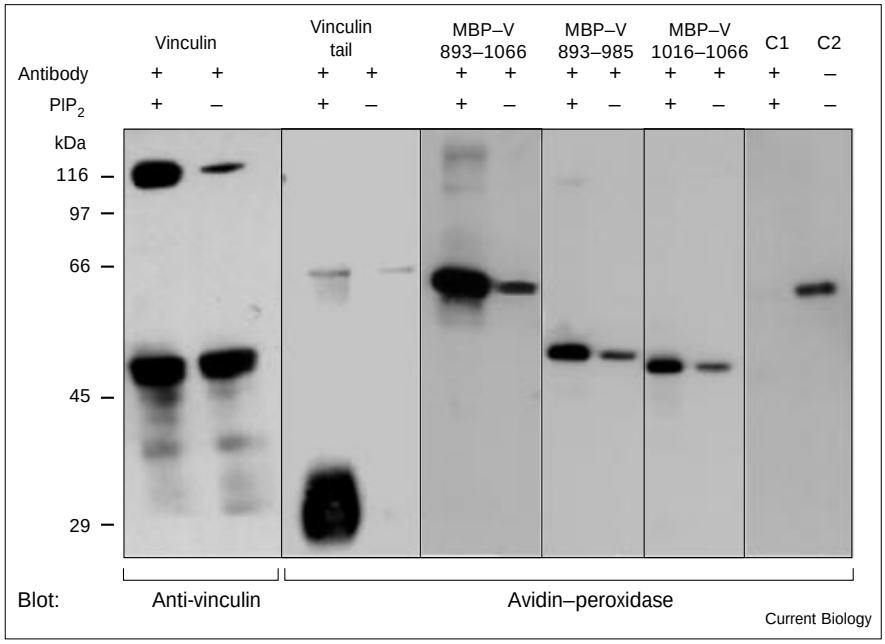


Figure 6



Mapping of PIP_2 binding sites within the vinculin tail. Full-length purified gizzard vinculin, biotinylated tail fragment, biotinylated MBP–vinculin tail fusion proteins (MBP–V; numbers refer to residues of the vinculin sequence), biotinylated MBP alone (C1) and MBP–V893–1066 alone (C2) were incubated with (+) or without (–) PIP_2 and immunoprecipitated with an anti-PIP_2 antibody. After SDS–PAGE, biotinylated samples were blotted and reacted with avidin–horseradish peroxidase (HRP), and vinculin was detected by an HRP-coupled anti-vinculin second antibody, as indicated. The intact vinculin, the tail fragment and the fusion proteins used were all precipitated by an anti-PIP_2 antibody, demonstrating that the vinculin tail sequence contains PIP_2-binding motifs in at least two sequence regions. A small amount of fusion protein is precipitated in the absence of both PIP_2 and the anti-PIP_2 antibody. This is due to non-specific binding of the protein to protein-A–Sepharose, as shown for MBP–V893–1066 in C2. The polypeptide present at a position of about 50 kDa in the vinculin sample is the heavy chain of the PIP_2 antibody labelled by the second, HRP-coupled antibody.

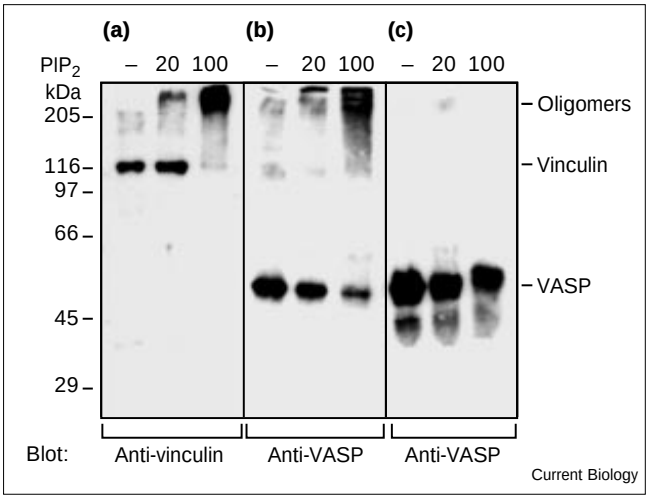
involved in the binding of PIP_2 to the tail domain. These are the same regions that are involved in the oligomerization of vinculin and in its binding to F-actin [26,34].

The interaction of VASP and vinculin is enhanced by PIP_2 binding to vinculin

Previous studies have identified VASP as a ligand for the proline-rich hinge in vinculin and suggested that VASP binds to the FP_4 motif (amino acids 842–846) within this region, which is flanked by additional prolines and acidic amino acids [8,9,18]. Encouraged by findings that several ligand interactions of vinculin are regulated by acidic phospholipids [27,29], we analyzed the influence of PIP_2 on the interaction of vinculin and VASP by chemical cross-linking (Figure 7). Equimolar amounts of both proteins were incubated in the presence or absence of PIP_2 and cross-linked with the zero-length cross-linker EDC. Reactions were stopped by boiling in SDS–PAGE sample buffer and the samples were analyzed by immunoblotting, using antibodies against vinculin (Figure 7a) and VASP (Figure 7b,c). Very little VASP was complexed with vinculin in the absence of PIP_2 (Figure 7a,b). When PIP_2 was added in a 20-fold molar excess, however, a significant amount of VASP was detected bound to vinculin at a high molecular weight position (Figure 7a,b). When PIP_2 was at a 100-fold molar excess over total protein, vinculin was shifted almost quantitatively to the position of the oligomers, and a major proportion of VASP comigrated with it (Figure 7a,b), indicating efficient complex formation in the presence of PIP_2. In control experiments performed without vinculin, VASP did not oligomerize, in either the absence or the presence of PIP_2 (Figure 7c). Thus, PIP_2 specifically enhances the interaction of vinculin with VASP.

Finally, we analyzed the regions in VASP that mediate complex formation with vinculin. The amino-terminal domain, a fragment consisting of the EVH1 domain plus the central proline-rich domain, and the carboxy-terminal EVH2 domain of VASP (see Figure 1 for explanation) were each produced as ^{35}S-labelled translation products and used as probes to assess binding to vinculin in dot overlay assays as described [34]. All three VASP fragments bound to vinculin, but only when vinculin had been activated by PIP_2 (data not shown). The overlay assay result for the amino-terminal domain was supported by cross-linking experiments: a recombinant protein consisting of the amino-terminal 151 amino acids of VASP (that is, the EVH1 domain with a 38 amino-acid extension into the central domain; see Figure 1) was used to characterize the complex formation with vinculin further. As shown in Figure 8, this fragment could be cross-linked to vinculin by EDC. In addition, we found that in mixtures of both proteins, this amino-terminal fragment could be coprecipitated with vinculin by anti-vinculin antibodies (data not shown). Hence, the interaction between vinculin and VASP might involve both the EVH1 and EVH2 domains.

Figure 7



The effect of PIP_2 on complex formation between VASP and vinculin, as demonstrated by chemical cross-linking. Vinculin and VASP were treated with EDC without PIP_2 (–) or with PIP_2 (the fold molar excess over protein is indicated above the lanes). One half of each aliquot was analyzed by immunoblotting with either **(a)** antibodies against the vinculin tail or **(b,c)** antibodies against VASP. In (c), VASP was treated with EDC in the absence of vinculin. Four times as much protein was loaded onto the gel in (c) compared with (a) and (b). VASP by itself, in the presence or absence of PIP_2, was not cross-linked. The positions of VASP, vinculin and hetero-oligomers are indicated.

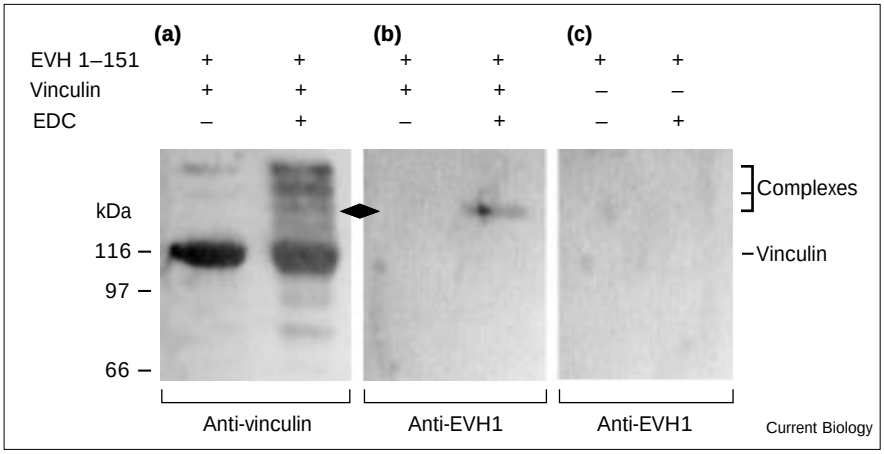
Discussion

In this paper, we have characterized the interaction of vinculin, a prominent structural component of focal contacts, and VASP, another constituent of these sites, which is at the same time a member of a signalling pathway [1]. We present evidence for the existence of vinculin–VASP complexes in living cells by immunoprecipitation experiments. The importance of the vinculin–VASP interaction for focal contact assembly is emphasized by the fact that both proteins are concentrated in the nascent focal contacts of spreading cells. Regarding the regions of the VASP sequence involved in the interaction with vinculin, the EVH1 domain has been characterized recently as a discrete functional module in VASP and related proteins, capable of interacting specifically with FP_4 motifs like those present in vinculin [18]. On the other hand, another study confined this interaction to a carboxy-terminal region of VASP contained in the EVH2 module [8]. On the basis of our results, we propose that both EVH1 and EVH2 VASP domains might contribute to vinculin binding.

Regarding vinculin, we found that its interaction with VASP *in vitro* is greatly influenced by the signalling molecule PIP_2, the intracellular level of which is elevated in several mammalian cell types during cell attachment [31,32]. PIP_2 enhancement of vinculin binding to VASP and its relative Mena has also been found independently from our studies by another group (A. Gilmore, L. Petch, K. Burridge and F. Gertler, unpublished observations, manuscript in preparation). As we observed that the PIP_2 effect is inhibited by PC, one has to assume that, in cells, vinculin has specific access only to microdomains in the plasma membrane that are enriched in PIP_2 but devoid of PC, or that PIP_2 is specifically delivered to vinculin from other proteins. Alternatively, the activating effect of PIP_2 described in this study is modulated in the living cell by additional factors, for example by phosphorylation [28]. PIP_2 binds to two discrete sequence stretches in the vinculin tail, thereby disrupting the head–tail interaction and inducing oligomerization of vinculin. Electron microscopic data have already demonstrated the formation of vinculin oligomers by tail–tail interactions [35], and photo-cross-linking studies with liposomes have suggested that vinculin oligomerizes as the result of its interaction with acidic phospholipids [36]. The PIP_2-binding sites, as identified in this study, coincide with two regions of predicted amphipathic helices [37] that are also involved in F-actin

Figure 8

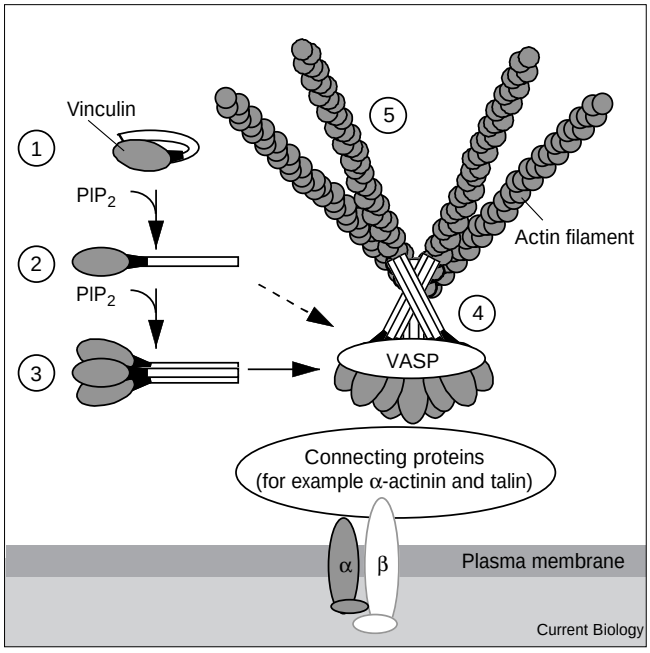
The interaction of the EVH1 domain of VASP with vinculin. Immunoblots of mixtures of a VASP amino-terminal fragment (EVH1 plus extension, amino-acid residues 1–151) with vinculin are shown, after cross-linking and incubation with either **(a)** an anti-vinculin antibody or **(b,c)** an antibody specific for the EVH1 domain of VASP. EVH1 is detected in one species of the vinculin oligomers formed by the cross-linker (b), but it is not detectable at this position after incubation with the cross-linker in the absence of vinculin (c). The double arrowhead indicates the cross-linked complex in (b).



binding [26,34]. It is conceivable that F-actin binding is due, at least in part, to the electrostatic attraction of the negative charges exposed on the surface of the actin filaments with the positively charged surface of these helices, although no prediction can be made as to which amino-acid residues within these regions might be involved in PIP_2 binding. We did not find any motif corresponding to the specific consensus sequence of basic amino acids identified in some PIP_2-binding proteins of the microfilament system, such as gelsolin and cofilin [38].

PIP_2 enhancement of VASP binding to the FP_4-containing hinge in vinculin, located amino-terminal of the PIP_2-binding tail fragment, might be explained by assuming that the VASP molecule can bind simultaneously to several closely spaced FP_4 motifs, such as those that might be exposed in a PIP_2-generated vinculin oligomer. Previous work has shown that in the absence of PIP_2, VASP and its relative Mena bind more strongly to proteins comprising several FP_4 motifs, such as zyxin and the bacterial protein ActA, than to purified vinculin [3,19]. Consistent with these *in vitro* results are data showing that in transfected cells the efficiency of VASP recruitment correlates with the number of FP_4 motifs in chimeric proteins comprising various vinculin and ActA sequences [39]. Furthermore, it has been shown recently that the proline-rich hinge region of vinculin containing the FP_4 motif is silent in intact vinculin but can be activated upon removing the vinculin tail sequence [40]. As discussed above, the EVH2 domain of VASP might also contribute to the binding of PIP_2-activated vinculin. Hence, PIP_2-induced vinculin activation might lead to an effective recruitment of vinculin oligomers and VASP to the focal contact site, a process that might be enhanced by the fact that VASP exists as a tetramer [4]. Tight bundling of the distal ends of actin filaments at focal adhesion sites by vinculin tails would then be a consequence of these events. The resulting arrangement of vinculin, VASP and F-actin is depicted schematically in Figure 9. In addition, the G-actin-binding protein profilin, which interacts with the proline-rich central region of VASP [7] and its relatives Mena and Evl [19] might deliver actin subunits to such complexes for rapid polymerization and elongation of the distal ends of actin filaments [9], a process imperative for rapid spreading and focal adhesion assembly. A large number of microfilament proteins in focal adhesion sites, such as vinculin, profilin, talin, α-actinin and gelsolin, bind to and are regulated by PIP_2 (see references within [13,41,42]). Changes in overall PIP_2 levels induced by cell attachment [32], as well as small, local variations in PIP_2 concentrations, achieved for example by activation of phospholipase Cγ1 [43] or the Rho GTPases [44], might have additive or even cooperative effects on focal adhesion assembly; this assembly might be regulated further by phosphorylation of vinculin [28] and VASP [1].

Figure 9



A possible model of the interactions between PIP_2, vinculin and VASP at focal adhesion sites. The cytoplasmic, closed form of vinculin (1) is opened and thus activated by PIP_2 (2) and induced to form oligomers via its tail domain (3). This model does not preclude other mechanisms that might additionally regulate vinculin and/or VASP activity, for example phosphorylation. Concomitantly or subsequently, vinculin forms complexes with VASP (4) in such a way that several FP_4 motifs exposed in the hinge region of vinculin oligomers bind to VASP. In the VASP–vinculin complexes, vinculin tails are engaged in bundling the distal ends of actin filaments (5), close to the plasma membrane. The vinculin heads and/or VASP can bind to proteins mediating the contact to integrins (αβ).

Our preliminary data suggest that VASP phosphorylation decreases the efficiency of complex formation with PIP_2-activated vinculin (our unpublished observations); binding of VASP to isolated proline-rich peptides of vinculin was not influenced by VASP phosphorylation, however [8]. Of the three residues in VASP that might be phosphorylated (Ser157, Ser239 and Thr278 [45]), none is located within the EVH1 domain that would bind to these peptides, but two are within the EVH2 domain. Hence, one might assume that binding to both domains is indeed necessary for complex formation and regulation. In platelets, VASP phosphorylation interferes with integrin (glycoprotein IIb/IIIa) binding to fibrinogen and, consequently, with platelet aggregation [46]. It remains to be seen whether a weakening of VASP–vinculin interactions induces such interfering effects on the activity of integrins in focal adhesions in general.

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Material and methods

Preparation of expression constructs

Avian vinculin cDNAs were prepared as described [26]. Specific cDNA sequences were amplified by PCR using the primers shown in Table S1 (see Supplementary material). To obtain coding sequences for proteins comprising MBP and fragments of the vinculin tail, the cDNAs were cloned into pMAL-c2 (New England Biolabs), using the restriction sites indicated in Table S1 (see above). The following three plasmids were obtained, designated according to the amino-acid residues of the vinculin tail: pMBP–V893–1066, pMBP–V893–985, pMBP–V1016–1066 (see Table S1). Fragments of cDNAs coding for the VASP EVH1 domain (residues 1–113), the EVH1 domain plus the proline-rich region (residues 1–225) and the EVH2 domain (residues 226–380) were amplified from a human VASP cDNA using specific primers (Table S1) and cloned into pcDNA3 (Invitrogen) to obtain pc-EVH1, pc-EVH1-PR and pc-EVH2, respectively, for *in vitro* translation. All constructs were verified by DNA sequencing.

Expression, purification and modification of recombinant proteins

E. coli DH5α cells were transformed with the vectors described above. Expression and purification of the various fusion proteins containing vinculin fragments were essentially as described [34]. VASP was produced as a recombinant protein with an amino-terminal His tag in *E. coli* (BL21(DE3)pREP4). Cells were grown to early log phase in 2 × YT supplemented with 0.2% glycerol, 50 mM potassium phosphate, pH 7.2, 5 μg/ml thiamine, 30 μg/ml L-proline, 25 μg/ml kanamycin and 100 μg/ml ampicillin and induced and further incubated after induction by 1 mM IPTG until early stationary stage. Bacterial sediments were resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 0.5 mM EDTA, 5% glycerol, 50 mM NaCl, 2 μg/ml leupeptin, 20 U/ml aprotinin, 5 mM benzamidine, 1 mM PMSF, 1 mg/ml lysozyme), incubated on ice for 30 min and lysed by sonication in the presence of 5 μg/ml DNAse I and 10 μg/ml RNase. After the addition of imidazole (final concentration 50 mM, pH 8.0) and Triton X-100 to 0.5%, the lysate was stirred on ice for 20 min. Subsequently, it was centrifuged (16,500 × g, 10 min), adjusted to 0.3 M NaCl and recentrifuged. The clarified extract was then incubated overnight at 4°C with 1 ml Ni–NTA agarose (Qiagen), equilibrated in the same buffer. The slurry was collected by centrifugation and extensively washed in lysis buffer (without EDTA and glycerol). The proteins were eluted in 50 mM sodium phosphate, pH 7.0, containing 0.3 M NaCl, 0.1 M EDTA, and dialyzed against cross-linking buffer (see below). An amino-terminal VASP fragment, slightly larger than the EVH1 domain (amino-acid residues 1–151), was expressed as a glutathione-S-transferase (GST) fusion protein. The GST moeity was removed using thrombin and the VASP fragment purified and characterized (T. Jarchau *et al.*, unpublished).

Purification of vinculin and proteolytic vinculin fragments

Turkey gizzard vinculin was purified as described [47] using an FPLC Q–Sepharose column (Pharmacia) instead of the DEAE column. Vinculin head and tail fragments were obtained by V8 protease digestion and purified essentially as described [24].

Preparation of phospholipid micelles

PIP_2, PS and PC (Boehringer Mannheim) were dissolved in chloroform (1 mg/ml), dried under vacuum for 20–30 min and resuspended in LP buffer (50 mM NaH_2PO_4, pH 7.2, 0.2 mM EGTA). For the preparation of mixed lipid micelles, the respective chloroform solutions were combined to yield the desired final concentrations. After sonication (5 × 1 min), the dispersion was centrifuged at 100,000 × g for 20 min and the supernatant was used in cross-linking and immunoprecipitation experiments.

Chemical cross-linking

Purified proteins or mixtures of two purified proteins were preincubated in the presence or absence of lipids at 37°C for 30 min. Cross-linking of proteins was achieved by incubating a single protein (25–100 pmol, 2.5 μM) or equimolar amounts of proteins (12.5–50 pmol, 1.2–1.4 μM) with 7 mg/ml N-hydroxysulfosuccinimide (NHS, Pierce) and 1.5 mg/ml EDC (Pierce) at 30°C for 5 min to 2 h in 50 mM NaH_2PO_4, pH 7.2, 0.2 mM EGTA. The reactions were stopped by adding SDS–PAGE sample buffer (375 mM Tris HCl pH 6.8, 2% SDS, 12% glycerol, 0.5 M β-mercaptoethanol, bromophenol blue). Samples were analyzed by SDS–PAGE and immunoblotting as described [26].

Protein biotinylation and immunoprecipitation

As some of the recombinant fusion proteins employed in this study showed an apparent molecular weight after SDS–PAGE similar to the heavy chain of the antibody used for immunoprecipitation, we used biotinylated proteins and HRP–avidin to detect the immunoprecipitated fusion proteins and the vinculin tail fragment. The proteins (0.1–0.3 mg/ml in 50 mM $NaHCO_3$ pH 8.3, 20 mM NaCl) were biotinylated essentially as described [34]. Biotinylation did not interfere with the biological activity of the probes, as judged by their ability to bind ligands as efficiently as their nonbiotinylated counterparts [34]. Full-length, purified vinculin, not biotinylated, and its proteolytically derived, biotinylated tail fragment and the biotinylated proteins (10 pmol, 0.7 μM) were incubated in the presence or absence of PIP_2 micelles (1 nmol, 70 μM) for 30–45 min at 37°C in LP buffer containing 20 mM β-mercaptoethanol. Samples were supplemented with 2% (w:v) BSA and incubated with an antibody against PIP_2 (DRG Instruments) for 1 h on ice. Immunocomplexes were precipitated by the addition of protein-A–Sepharose (Sigma). After another incubation for 1 h on ice, and 15 sec centrifugation at 10,000 × g, pellets were collected and washed four times with TBST (20 mM Tris HCl pH 7.6, 150 mM NaCl, 0.1% (v:v) Tween-20, 20 mM β-mercaptoethanol) and subjected to SDS–PAGE. Precipitated recombinant proteins were identified by incubating the gels with avidin–HRP, followed by enhanced chemiluminescence. For the detection of purified vinculin, a vinculin-tail-specific antibody and a second HRP-coupled antibody were used.

Immunoprecipitation of protein complexes after in situ *cross-linking*

In situ cross-linking was performed as described [48,49]. Briefly, 2.5×10^6 HeLa cells were grown on 10 cm dishes in DMEM with 10% FCS. They were then washed twice with PBS and incubated at room temperature for 30 min in 2 ml PBS per dish, containing the membrane-permeable cross-linker DSP (final concentration 0.5 mM). After two subsequent washing steps in PBS, excess cross-linker was quenched with 0.2 M glycine in 2 ml PBS per dish. Finally, the cells were again washed with PBS and incubated for 30 min at 4°C in 1 ml per dish RIPA buffer (50 mM Tris, pH 7.2, 1% (v:v) Triton X-100, 0.25% desoxycholate, 1 mM EGTA, 150 mM NaCl, 20 mM glycine, 2.5 mM sodium azide, 1 μM pepstatin A, 80 μM pefabloc SC, 0.46 μM aprotinin) for lysis. Control samples, not treated with cross-linker, were obtained by directly lysing cells in RIPA buffer. Cellular material was then scraped off the dish with a rubber policeman, homogenized by pipetting and centrifuged at 15,000 × g for 10 min at 4°C. Monoclonal antibodies against human vinculin or VASP were added to the supernatants (5 μl anti-vinculin, 100 μl of a mixture of two monoclonal VASP antibodies, see below) and the samples were incubated for 16 h at 4°C. Then 50 μl of a 50% slurry of protein-G–Sepharose preblocked with 2% BSA in RIPA buffer was added to the samples, which were then further incubated under stirring at 4°C for 1 h. The Sepharose beads were then collected by centrifugation, washed twice in RIPA buffer and once with PBS before the samples were boiled in SDS sample buffer including 20% β-mercaptoethanol, to cleave the cross-linker. Finally, samples were analyzed by SDS–PAGE and immunoblotting.

Antibodies

Monoclonal antibodies specific for the vinculin head (15E7) and tail (4E7) fragments were raised against avian vinculin and characterized in

our laboratory. A monoclonal antibody against human vinculin was from BIOMOL. The monoclonal antibodies against human VASP, including some which were specific for either the EVH1 or EVH2 domains of VASP, were a kind gift of J. Wehland. Goat serum raised against a VASP-specific peptide (amino-acid residues 339–357) was from Santa Cruz Biotechnology. A monoclonal antibody against PIP_2 was from DRG Instruments. Secondary antibodies for immunoblots were HRP-coupled goat anti-mouse, alkaline-phosphatase-coupled rabbit anti-mouse, or rabbit anti-goat IgG, respectively, from Sigma. For immunofluorescence, goat anti-mouse Fab conjugated with FITC (Dianova) and goat anti-mouse IgG conjugated with TRITC (Sigma) were used.

Localization of VASP and vinculin in cells.

Non-confluent cultures of HeLa cells were removed from the dish. Cells (5×10^5) were reseeded on collagenized glass coverslips and returned to the incubator for 30–60 min. Cells were then fixed with 3.7% formaldehyde, extracted in 0.2% Triton X-100 and processed for double immunofluorescence. They were incubated with the monoclonal antibody against human vinculin, followed by FITC-conjugated goat anti-mouse Fab, and subsequently with a monoclonal anti-VASP and goat anti-mouse IgG conjugated with TRITC. Images were obtained by using either a conventional light microscope (Zeiss) equipped with epi-fluorescence and conventional photography, or a confocal laser scanning microscope (Noran Instrument) with the corresponding image processing equipment.

Supplementary material

A table of the primers used for PCR of vinculin constructs is published with this paper on the internet.

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